

# Expression of *RHD* and *RHCE* Gene Products Using Retroviral Transduction of K562 Cells Establishes the Molecular Basis of Rh Blood Group Antigens

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Retroviral-mediated gene transfer using cDNA transcripts of the *RHD* and *RHCE* genes resulted in the isolation of K562 clones expressing D and G or c and E antigens, respectively. These results represent the first direct demonstration that the *RHD* gene encodes the D and G antigens and the *RHCE* gene encodes the c and E antigens. Both c and E antigens

were expressed after transduction of K562 cells with a single cDNA, indicating that the c antigen does not arise by alternative splicing (exon skipping) of the product of the *RHCE* gene, as has been suggested.

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THE RH SYSTEM is the most complex of the 23 blood group systems found on human red blood cells.<sup>1</sup> Antibodies to Rh system antigens (especially anti-D) are of clinical significance because they may cause hemolytic disease of the newborn or transfusion reactions.<sup>2</sup> In recent years, there have been considerable advances in our understanding of the biochemistry and genetics of Rh antigens.<sup>3-5</sup> Available evidence suggests that the Rh antigens result from at least two highly homologous genes (*RHD* and *RHCE*) located at chromosome 1 p34-p36. The *RHD* gene is deleted in most white individuals who lack the D antigen.<sup>6</sup> The *RHCE* gene gives rise to the allelic antigens C/c and E/e. It has been proposed that C/c and E/e are located on different polypeptides which arise by alternative splicing of the primary transcript of the *RHCE* gene.<sup>7,8</sup>

Formal proof that the *RHD* and *RHCE* genes encode for the antigens of the Rh system and that alternative splicing of the *RHCE* gene product gives rise to separate polypeptides with C/c or E/e antigens, respectively, has been lacking because previous attempts to express these genes in eukaryotic cells have been unsuccessful.<sup>9,10</sup>

In this report we describe the use of retroviral gene transfer to generate stable clones of K562 cells expressing the D and G, or c and E blood group antigens. The results provide the first direct evidence that the putative *RHD* gene gives rise to D and G antigens and that the putative *RHCE* gene gives rise to c and E antigens. However, the results refute the hypothesis that the c and E antigens arise by alternative splicing of the product of the *RHCE* gene.

## MATERIALS AND METHODS

*Cloning of Rh D and cE cDNAs into the pBabe puro retroviral vector.* The Rh cE cDNA (1,463 bp) was cloned into Bluescript vector as previously described.<sup>11</sup> It contained 41 nucleotides of 5' and 171 nucleotides of 3' noncoding sequence and terminated with an A<sub>16</sub> tract. The Rh D cDNA (1,359 bp) was cloned into pCRII vector (Invitrogen, San Diego, CA) after reverse transcriptase-poly-

merase chain reaction (RT-PCR) of total RNA purified from reticulocytes<sup>12</sup> of Rh phenotype CDe. RT-PCR was performed in a Perkin-Elmer DNA thermal cycler (Perkin-Elmer, Norwalk, CT) using avian myeloblastosis virus reverse transcriptase, oligo dT<sub>20</sub> (Pharmacia, Uppsala, Sweden) and Taq polymerase (Perkin-Elmer) essentially as described.<sup>13</sup> The primers were based on the cE cDNA 5' and 3' noncoding nucleotides -32 to -5 (exon 1 sense) and 1,300 to 1,327 (exon 10 anti-sense), respectively. Transformation of *Escherichia coli* XL-1 Blue competent cells (Invitrogen) with pCRII and subsequent vector DNA preparation (Qiagen, Dorking, UK) was done according to the manufacturers' instructions. A colony of cells containing only D cDNA was selected following the sequencing of several vector/insert DNA preparations. The D cDNA sequence differed from that described<sup>14</sup> in that it contained a C<sub>634</sub> to G (Ile<sub>219</sub> to Met) change in the coding region (previously reported<sup>15</sup>) and an A<sub>1272</sub> to G change in the 3' noncoding region. The Fy<sup>b</sup> cDNA (1,062 bp) was cloned into Bluescript vector (Stratagene, La Jolla, CA) following amplification of genomic DNA from an Epstein-Barr virus (EBV) lymphoblastoid cell line derived from an individual of phenotype Fy(a-b+) using primers based on the 5' and 3' Fy<sup>b</sup> noncoding regions as described.<sup>16</sup>

The D and cE cDNAs were subcloned separately into the pBabe puro retroviral vector (kindly provided by Dr H. Land, ICRF, London, UK) using the *EcoRI* restriction site.<sup>17</sup> The Fy<sup>b</sup> cDNA was subcloned into pBabe puro using the *BamHI* and *SalI* restriction sites. The pBabe puro vector is based on the Moloney murine leukaemia virus (MoMuLV). Expression of inserted genes is driven by the MoMuLV long terminal repeat while the puromycin resistance gene is expressed from the SV40 early promoter 3' of the cloning site. Vector DNA preparation was as described above. Correct Rh cDNA orientation in pBabe was established by *BamHI* and *KpnI* restriction and DNA sequence analysis.

*Transfection of the packaging cell line and retroviral supernatant production.* Amphotropic retroviral packaging cells, GP + env AM12,<sup>18</sup> from Genetix Pharmaceuticals (Rye, NY), were cultured in Iscove's modified Eagle's medium supplemented with 10% fetal bovine serum (IMEM/FBS) and incubated at 37°C in a 5% CO<sub>2</sub> humidified incubator. Cells (5 × 10<sup>6</sup>) were transfected with pBabe constructs (10 to 25 µg) using calcium phosphate precipitation essentially as described.<sup>13</sup> Four hours after the addition of DNA the culture medium was replaced with 15% glycerol in phosphate-buffered saline (PBS) for 3 minutes and this in turn was replaced with IMEM/FBS. After 2 days the medium was replaced with fresh IMEM/FBS supplemented with puromycin (3 µg mL<sup>-1</sup>; Sigma, St Louis, MO). Approximately 2 weeks later individual puromycin resistant colonies were transferred to culture flasks using trypsin (Sigma) to detach the cells. The supernatants were collected from near-confluent cells incubated overnight at 33°C, filtered (0.45 µm), and stored frozen.

*Retroviral transduction of K562 cells.* K562 cells were obtained from the European Collection of Animal Cell Cultures (Porton Down, Salisbury, Wiltshire, UK). Cells (10<sup>6</sup>) were incubated at 37°C in IMEM/FBS supplemented with viral supernatant (1.0% vol/vol) and polybrene (8 µg mL<sup>-1</sup>; Sigma). After 4 hours the cells were centrifuged at 400g for 5 minutes and resuspended in IMEM/FBS.

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Two days later the cells were recultured in IMEM/FBS supplemented with puromycin ( $3 \mu\text{g mL}^{-1}$ ) and transferred to a 96-well microplate (0.2 mL/well). After 2 to 3 weeks puromycin-resistant stable-transduced clones from wells containing only a single discrete colony were transferred to 24-well plates and expanded before flow cytometric analysis. The clones exhibiting the highest levels of Rh expression were selected using BRIC 69 and those with the highest levels of  $\text{Fy}^b$  expression using CBC-512 (vide infra).

**Antibodies and red blood cells.** BRIC 69 is a murine monoclonal antibody that binds to Rh polypeptides.<sup>19</sup> Murine monoclonal anti-Fy3, (CBC-512, IgG) and human monoclonal anti-E (H4-1 G-4, IgG) were provided by Dr M. Uchikawa (Japanese Red Cross, Shibuya-ku, Japan). Purified human monoclonal anti-D, BRAD 5 (IgG1<sup>20</sup>) was used at  $50 \mu\text{g/mL}$  (diluted in PBS/1% bovine serum albumin [BSA], 0.1%  $\text{NaN}_3$  [PBS-A]). Human monoclonal anti-C (MS252, IgG3), anti-c (MS47, IgG3), anti-e (MS70, IgG3), and Anti-G (MS1, IgG3) were provided by Dr K. Thompson (IGRI, Oslo, Norway). Red blood cells of known Rh phenotype were available from the National Blood Service, Bristol.

**Flow cytometric analysis.** K562 clones transduced with D cDNA (K562/D) or cE cDNA (K562/cE) were tested for antigen expression by flow cytometry (FACStar Plus; Becton Dickinson, Mountain View, CA). Mean fluorescence intensity (FLI) was used as a measure of antibody binding. The specificity of the antibodies used was confirmed using red blood cells of the appropriate phenotype (data not shown). A K562 clone transduced with cDNA corresponding to the  $\text{Fy}^b$  blood group gene (K562/ $\text{Fy}^b$ ) and untransduced K562 cells were used as controls. K562 cells ( $2 \times 10^5$ ) or red blood cells (0.5% suspension) in PBS-A (50  $\mu\text{L}$ ) were incubated with antibody (50  $\mu\text{L}$ ) for 1 hour at  $37^\circ\text{C}$ . Cells were washed once in PBS-A and incubated with (Fab')<sub>2</sub> fragments of rabbit-antihuman IgG or rabbit-antimouse IgG affinity-purified fluorescein isothiocyanate (FITC)-labeled (1/20, 50  $\mu\text{L}$ , DAKO, Glostrup, Denmark) for 1 hour at room temperature. The sample volume was adjusted to 300  $\mu\text{L}$  with PBS-A before analysis.

**mRNA preparation and RT/PCR amplification of pBabe specific sequences from K562 cells.** Oligo d(T<sub>12-18</sub>) magnetic beads were used to prepare mRNA according to the manufacturers instructions (Dynal, Oslo, Norway). Synthesis of cDNA was from 1  $\mu\text{g}$  of mRNA as described above using oligo d(T<sub>12-18</sub>) (Pharmacia). Approximately 100 ng of cDNA was used as the template for PCR ( $94^\circ\text{C}$  1 minute,  $60^\circ\text{C}$  1 minute,  $72^\circ\text{C}$  2.5 minutes, 35 cycles) with 50 pmol of each primer. Two pairs of primers were used. One set (P1 and P2) corresponded to pBabe sequences 5' and 3' of the cloning site (P1 sense 5'-CCC TTT ATT CAG CCC TCA CTC CT-3' and P2 anti-sense 5'-CCC TAA CTG ACA CAC ATT CCA CAG-3'), respectively. The second set consisted of a primer (P3) which corresponded to pBabe sequence 5' of the insert site (P3 sense 5'-GCC TCG ATC CTC CCT TTA TCC-3') and the antisense primer to cE cDNA 3' noncoding region (exon 10 antisense, described above).

**Sequencing of cDNA.** Amplified Rh-specific PCR products were sequenced using dye-labeled terminator chemistry on a 373A Applied Biosystems automated sequencer (Warrington, UK). Set 1 and Set 2 primers and primers complementary to sequences common to both the cE and D coding regions were used for sequencing.

**Rh typing of genomic DNA.** Genomic DNA was prepared using the proteinase K/sodium dodecyl sulfate (SDS)/EDTA method essentially as described.<sup>13</sup> Rh D-specific PCR was performed using primers specific for intron 4 and exon 10 of the *RHD* gene.<sup>21,22</sup> Allele-specific PCR for c and E alleles was performed as described.<sup>23</sup>

## RESULTS

**Flow cytometric analysis of K562 clones transduced with putative D and CE genes.** K562/D and K562/cE clones,

prepared as described in Materials and Methods, were tested for Rh antigen expression by flow cytometric analysis. A K562/ $\text{Fy}^b$  clone and untransduced K562 cells were used as controls. The selected clones were examined using human monoclonal antibodies to Rh system antigens (D, C, E, c, e, and G). The results for a K562/D clone are shown in Fig 1A and Table 1. Anti-D and anti-G bound much more strongly to this clone than to the K562/ $\text{Fy}^b$  clone with increases in mean fluorescence intensity (FLI) from 3.3 to 15.2 and 4.5 to 11.0 for anti-D and anti-G, respectively. Antibodies to C, E, c, and e antigens did not detect comparable changes in antigen expression, although anti-c and anti-e did show very minor increases in binding to K562/D cells (Table 1).

The results obtained for a K562/cE clone are shown in Fig 1B. Anti-c and anti-E bound much more strongly to K562/cE cells than to K562/ $\text{Fy}^b$  cells with increases in FLI from 4.7 to 24.4 (anti-c) and from 2.4 to 38.8 (anti-E), respectively (Table 1). Antibodies to D, G, C, and e antigens did not detect comparable changes in antigen expression, although anti-D, anti-G, and anti-e did show very minor increases in binding to K562/cE cells.

The FLI of Rh antibody binding to untransduced K562 cells and K562/ $\text{Fy}^b$  cells was compared with that of tissue culture medium (TCM, Table 1). Antibodies to C, E, and e antigens gave almost identical FLI values to TCM with both untransduced K562 cells and K562/ $\text{Fy}^b$  cells. Antibodies to D, G, and c antigens gave slightly higher FLI values than TCM with both cell preparations (Table 1).

In a separate experiment, murine monoclonal anti-Fy3 gave almost identical FLI values to those obtained with TCM with K562/D, K562/cE, and untransduced K562 cells (range 2.2 to 2.5) while K562/ $\text{Fy}^b$  gave an FLI value of 24.2 (data not shown).

**Demonstration of D and cE mRNAs in the K562/D clone and the K562/cE clone, respectively.** The presence of mRNAs arising from retroviral transduction in K562/D and K562/cE clones was determined by isolation of mRNA followed by RT-PCR and DNA sequence analysis. Two sets of oligonucleotide primers were used. Primer set 1 (P1 and P2, see Materials and Methods) was specific for vector (pBabe puro) sequences flanking the multiple cloning site. Using these primers a product of 1.55 kb was amplified from K562/D cDNA and a product of 1.15 kb from the K562/ $\text{Fy}^b$  cDNA. No products were obtained from untransduced K562 cells or from the K562/cE clone (Fig 2). Primer set 2 (P3 and an Rh specific exon 10 antisense primer, see Materials and Methods) amplified a product of 1.5 kb from the K562/cE cDNA and from the K562/D cDNA, but no products were obtained using cDNA from untransduced K562 cells or the K562/ $\text{Fy}^b$  clone (Fig 2).

The PCR product of 1.55 kb obtained from K562/D cDNA with primer set 1 was sequenced (see Materials and Methods). It contained the expected 5' and 3' pBabe vector flanking sequences and a D cDNA sequence (from nucleotide -32 to 1327) identical to that of the cDNA originally subcloned into pBabe. The PCR product of 1.5 kb obtained from K562/cE cDNA with primer set 2 was sequenced (see Materials and Methods). It contained the expected pBabe 5' flanking sequence and a cE cDNA sequence corresponding

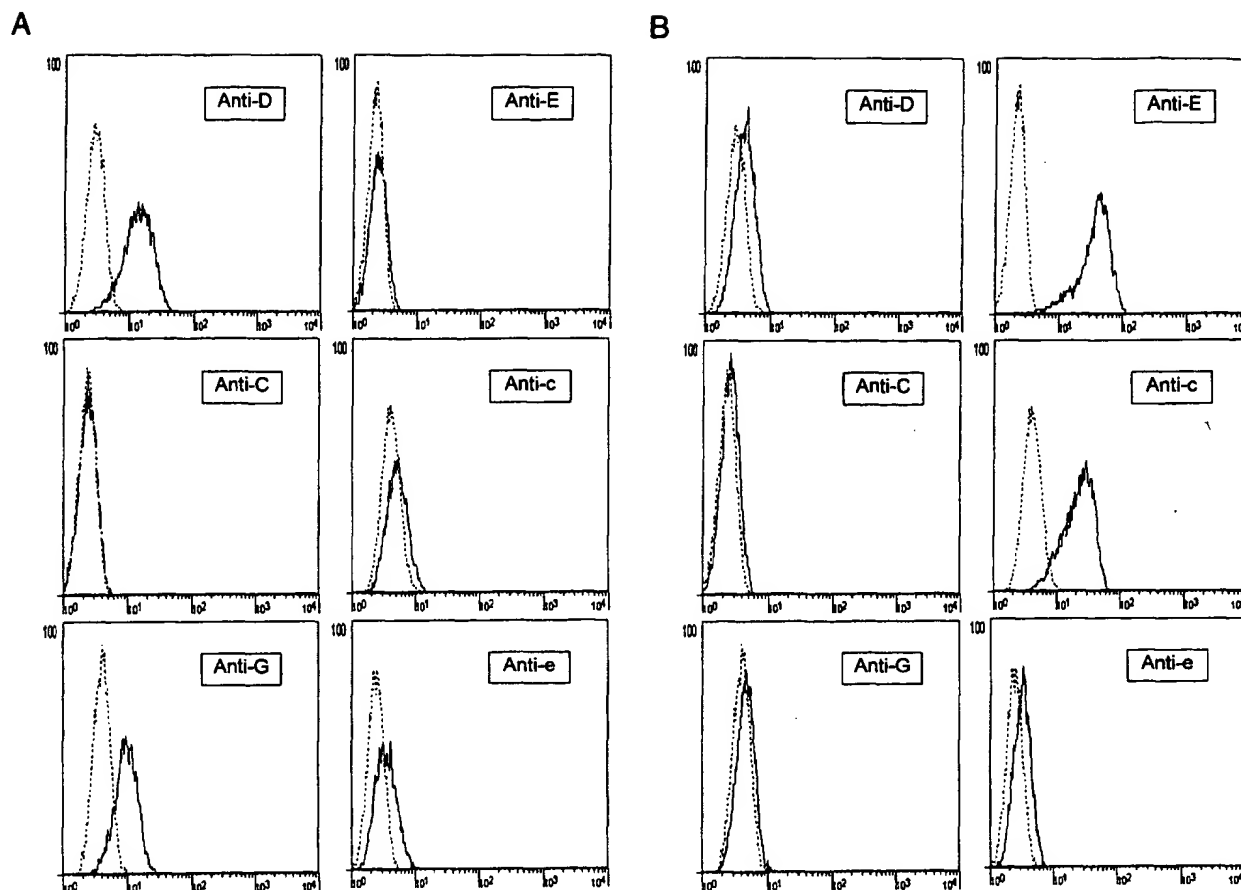


Fig 1. Flow cytometric analysis of transduced K562 cells using monoclonal Rh antibodies. (A) (—), K562/D; (---), K562/Fy<sup>b</sup>. (B) (—), K562/cE; (---), K562/Fy<sup>b</sup>. The number of cells is plotted on the ordinate and the fluorescence intensity on the abscissa. Experimental details are in Materials and Methods.

to nucleotides -41 to 1327 of that originally subcloned into pBabe. A product of the expected size<sup>16</sup> (1.15 kb) was amplified from the K562/Fy<sup>b</sup> cDNA with primer set 1 (Fig 2).

**Rh typing of genomic DNA from untransduced and transduced K562 cells.** Analysis of genomic DNA derived from untransduced K562 cells, K562/D, and K562/Fy<sup>b</sup> showed the presence of D- and c- (data not shown), but not E-specific sequences (Fig 3). The presence of an E-antigen-specific product (149 bp) in genomic DNA derived from the K562/

cE clone established that integration of the cE cDNA had occurred (Fig 3).

## DISCUSSION

The purpose of the present work was to investigate K562 cells as a model in vitro system for the study of *RH* gene expression. Previous attempts to express Rh cDNAs by transfection of K562 cells have met with little success.<sup>9,10</sup> In the present study we have used retroviral gene transfer to transduce K562 cells with cDNAs corresponding to the putative *RHCE* gene (syn RhIXb<sup>24</sup>; Rh30A<sup>11</sup>), and the putative *RHD* gene (syn RhXIII<sup>14</sup>; Rh13<sup>15</sup>; RhPII<sup>25</sup>). The levels of D, c, E, C, e, and G antigen expression on these transduced cells were compared with cells transduced with cDNA corresponding to Fy<sup>b</sup> glycoprotein gene<sup>16,26-28</sup> and with untransduced K562 cells. K562/D cells gave much higher FLI values with anti-D and anti-G in comparison with K562/Fy<sup>b</sup> cells (Fig 1A), K562/cE cells, or untransduced K562 cells (Table 1). In contrast, K562/cE cells gave much higher FLI values with anti-c and anti-E than the other cells tested (Fig 1B and Table 1). The levels of Rh antigen expression on K562/Fy<sup>b</sup> cells were virtually identical to those on untransduced cells (Table 1). The expression of D, c, and E antigens

Table 1. Rh Antigen Expression on Untransduced K562 Cells and K562 Clones Transduced With D, cE, and Fy<sup>b</sup> cDNA

Cells	TCM	Rh Antibody					
		Anti-D	Anti-C	Anti-G	Anti-E	Anti-c	Anti-e
K562	2.8	3.8	2.8	4.2	2.5	5.2	2.8
K562/Fy <sup>b</sup>	2.8	3.3	2.5	4.5	2.4	4.7	2.6
K562/D	2.7	15.2	2.6	11.0	2.7	6.1	3.8
K562/cE	2.8	4.5	2.9	5.9	38.8	24.4	3.4

Mean fluorescence intensity (FLI) was used as a measure of antibody binding. Antibody binding is compared with background fluorescence obtained when the cells were incubated in tissue culture medium (TCM).

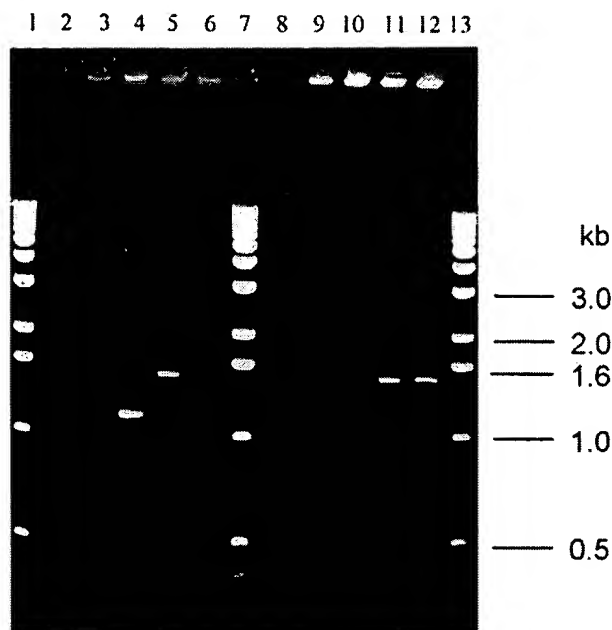


Fig 2. Demonstration of D and cE mRNAs in K562/D and K562/cE cells. mRNA preparation and RT-PCR was as described in Materials and Methods. Lanes 2 through 6, PCR performed with set 1 primers; lanes 8 through 12, PCR performed with set 2 primers; 2, 8, no template control; 3, 9, cDNA from untransduced K562 cells; 4, 10, cDNA from K562/Fy<sup>b</sup> cells; 5, 11, cDNA from K562/D cells; 6, 12, cDNA from K562/cE cells; 1, 7, 13, 1-kb ladder (GIBCO-BRL, Paisley, UK).

that we observed is consistent with previous predictions based on sequence analysis of putative RH genes in individuals of known Rh phenotype.<sup>7</sup>

The G antigen is an Rh antigen that is expressed on red blood cells carrying a D and/or C antigen.<sup>29</sup> Our experiments involving expression of the *RHD* gene showed that the same gene also gives rise to the G antigen (Fig 1A). This observation is consistent with recent studies<sup>30</sup> suggesting that G antigen expression is dependent on the amino acid sequence deriving from exon 2 of the *RHD* gene.

Previous studies have indicated that K562 cells express low levels of mRNA corresponding to Rh polypeptides and that Rh antigens can be detected on the cell surface.<sup>31,32</sup> Our flow cytometric results suggest the possibility that there are

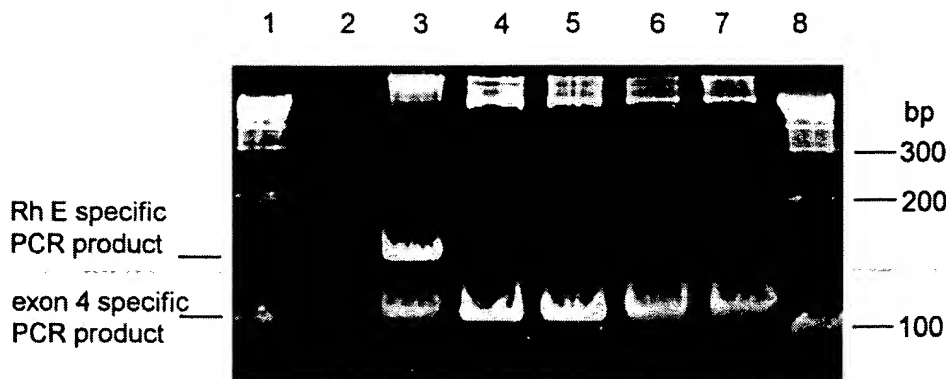
low levels of Rh antigen expression on the untransduced K562 cells used in this study (compare FL1 in the presence of TCM with that in the presence of Rh antibodies, Table 1). In addition, the slightly increased binding of anti-c and anti-e to K562/D cells and anti-D, anti-G, and anti-e binding to K562/cE cells (Table 1) may indicate enhanced expression of existing Rh antigens on K562 cells after transduction with Rh cDNAs. However, the increases in antibody binding are small and may be artifactual. K562 cells express FcRII receptors that are known to bind oligomeric human antibodies.<sup>33</sup> Therefore, it is possible that the weak binding of some human monoclonal antibodies to K562 cells is nonspecific, a problem we and others have found associated with polyclonal alloimmune sera.<sup>34</sup>

Confirmation that the cDNA corresponding to *RHD* and *RHCE* genes had been inserted into the K562 clones and was responsible for antigen expression was obtained by amplification and sequencing of pBabe-specific cDNA following RT-PCR with purified mRNA. Primers specific for pBabe amplified only the D-specific cDNA from cells transduced with D cDNA. The amplification of pBabe/cE specific cDNA from K562/cE cells required a 5' pBabe primer and an anti-sense primer to the cE cDNA 3' noncoding region. This suggests the pBabe/cE RNA transcript was terminated by the poly A tract at the 3' end of the cE cDNA and did not include pBabe sequence 3' of the cE cDNA insert. RNA transcripts generated from the pBabe template are usually terminated by the pBabe Poly A signal positioned approximately 2.5 kb 3' of the cloning site.

Typing of genomic DNA from untransduced K562 cells provided evidence for D- and c- but not E-antigen-specific sequence (vide supra). Confirmation that the putative *RHCE* gene product was inserted into the genome of the transduced K562/cE cells was obtained by the use of allele-specific PCR which demonstrated the presence of E-antigen-specific sequence (Fig 3). It was not possible to analyze genomic DNA for C- and e-antigen-specific sequences in the presence of the *RHD* gene because the *RHD* gene contains homologous sequences. Differences in the conformation of the proteins encoded by the *RHD* and *RHCE* genes are thought to ensure that the C and e antigens are only expressed when encoded by the *RHCE* gene.<sup>4</sup>

The Rh polypeptides encoded by the *RHD* and *RHCE* genes are known to associate with a glycoprotein of  $\approx 50$  kD

Fig 3. Rh E typing of genomic DNA from untransduced and transduced K562 cells. PCR was performed using primer pairs specific for E and exon 4 of the *RHCE* gene as described in Materials and Methods. Lane 2, no template control; 3, gDNA from the lymphocytes of an individual of Rh phenotype cE; 4, gDNA from untransduced K562 cells; 5, gDNA from K562/Fy<sup>b</sup> cells; 6, gDNA from K562/D cells; 7, gDNA from K562/cE cells; 1, 8, 100-bp ladder (GIBCO-BRL).



(Rh glycoprotein<sup>35,36</sup>) and evidence obtained from analysis of the rare Rh<sub>null</sub> erythrocytes which lack all Rh antigens has led to suggestions that other red blood cell proteins (glycophorin B, CD47, LW glycoprotein, FY glycoprotein) are also part of an 'Rh complex.' Available evidence indicates that untransduced K562 cells express Rh glycoprotein<sup>32</sup> (and unpublished observations, November 1992), CD47,<sup>37</sup> and glycophorin B,<sup>38</sup> but not LW<sup>37</sup> or FY,<sup>26</sup> and therefore K562 cells provide an attractive model for the study of Rh antigen expression in vitro. However, previous attempts to express Rh antigens in K562 cells using plasmid expression vectors have been unsuccessful.<sup>9,10</sup> Our results indicate that the use of retroviral delivery of the gene is of critical importance in achieving expression of Rh antigens on K562 cells.

We considered the possibility that the process of retroviral transduction may, of itself, activate existing *RH* genes. The results with K562/Fy<sup>b</sup> clones show quite clearly that we are not observing a phenomenon related to the retrovirus but the genuine expression of D and cE cDNAs inserted into the genome of K562 cells. These results provide the first direct evidence that the proposed *RHD* and *RHCE* gene products do indeed code for the D and cE antigens, respectively. The results also suggest that FY is not vital for the assembly and expression of Rh because D, G, c, and E antigens are expressed in its absence.

Our experiments involving expression of the cE cDNA are relevant to the hypothesis that C/c and E/e antigens are expressed on different proteins which derive from alternative splicing of the product of the *RHCE* gene.<sup>7</sup> Mouro et al<sup>7</sup> proposed that the full-length mRNA product of the *RHCE* gene produces a protein that expresses E or e antigen but not C or c antigen and that spliceforms of this mRNA, giving rise to products that lack exon 5 (which encodes the critical residues for E/e antigen activity), are translated into proteins that only express C/c antigens.

The validity of this hypothesis has been questioned since the spliceforms have not been detected in Northern blotting studies and it has not been established that they give rise to proteins which are expressed in a stable form in the red blood cell membrane.<sup>5,39</sup> Our results clearly show that alternative splicing of hnRNA is not a prerequisite for c antigen expression and suggest that a single polypeptide is able to express both c and E antigens.

Progress in elucidating the molecular basis of the Rh system antigens, the role of Rh-associated proteins in antigen expression, and the assembly and transport of the 'Rh complex' to the membrane has been severely hampered by the lack of an in vitro expression system. These results suggest that retroviral-mediated gene transfer into K562 cells provides such an in vitro system.

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